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Effects of Shh and Noggin on neural crest formation demonstrate that BMP is required in the neural tube but not ectoderm

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SUMMARY

To define the timing of neural crest formation, we challenged the fate of presumptive neural crest cells by grafting notochords, Sonic Hedgehog- (Shh) or Noggin-secreting cells at different stages of neurulation in chick embryos. Notochords or Shh-secreting cells are able to prevent neural crest formation at open neural plate levels, as assayed by DiI-labeling and expression of the transcription factor, *Slug*, suggesting that neural crest cells are not committed to their fate at this time. In contrast, the BMP signaling antagonist, Noggin, does not repress neural crest formation at the open neural plate stage, but does so if injected into the lumen of the closing neural tube. The period of Noggin sensitivity corresponds to the time when BMPs are expressed in the dorsal neural tube but are down-regulated in the non-neural ectoderm. To confirm the timing of neural crest formation, Shh or Noggin were added to neural folds at defined times in culture. Shh inhibits neural crest production at early stages (0-5 hours in culture), whereas Noggin exerts an effect on neural crest production only later (5-10 hours in culture). Our results suggest three phases of neurulation that relate to neural crest formation: (1) an initial BMP-independent phase that can be prevented by Shh-mediated signals from the notochord; (2) an intermediate BMP-dependent phase around the time of neural tube closure, when BMP-4 is expressed in the dorsal neural tube; and (3) a later pre-migratory phase which is refractory to exogenous Shh and Noggin.

Key words: Neural crest cells, Notochord, *Slug*, Neural plate, Commitment, Chick

INTRODUCTION

The neural tube becomes polarized dorsoventrally such that different cell types differentiate in dorsal versus ventral regions. Dorsal structures include the roof plate and neural crest, whereas ventral structures include the floor plate and motor neurons. A number of genes are expressed in precise domains of the neural tube; for example the secreted factors like *Wnt-1* , *Wnt-3a* (Wilkinson et al., 1987; Roelink and Nusse, 1991; Hollyday et al., 1995), *dorsalin-1* (Basler et al., 1993), BMP-4 and BMP-7 (Liem et al., 1995) and transcription factors such as *Pax-3* (Goulding et al., 1991, 1993) and *Slug* (Nieto et al., 1994) are expressed in the dorsal neural tube whereas *HNF-3β* (Monaghan et al., 1993), *Sonic hedgehog* (*Shh*; Echelard et al., 1993) and various LIM domain-containing transcription factors (Tanabe and Jessell, 1996) are expressed ventrally.

What mechanisms establish polarity within the neural tube? The notochord is one source of polarizing signals: a notochord grafted lateral to the neural tube induces the formation of ectopic floor plate cells and motor neuron pools (van Straaten et al., 1988, 1989; Smith and Schoenwolf, 1989; Placzek et al., 1990; Yamada et al., 1991), an effect that is mediated by Sonic hedgehog (Shh; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1995; Marti et al., 1995a,b). One possibility is that the notochord is responsible for polarizing the entire neural tube, such that those cells furthest from the notochord develop, by default, into dorsal cell types. This seems unlikely, however, since ventral neural tube cells cannot form neural crest when cultured alone. Work from our laboratory (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995) has shown that, in the chick embryo, an interaction between the neuroepithelium and the adjacent non-neural ectoderm results in the formation of neural crest cells and expression of some genes such as *Slug*, *Wnt-1* and *Wnt-3a*. Two members of the transforming growth factor superfamily, *BMP-4* and *BMP-7*, are expressed in the superficial ectoderm and subsequently in the dorsal neural tube (Watanabe and Le Douarin, 1996) and have been shown by Liem et al. (1995) to mimic the epidermis in its inducing ability.

When during neurulation does neural crest formation occur? Single cell lineage analyses of the ectoderm and its derivatives (Bronner-Fraser and Fraser, 1988, 1989; Selleck and Bronner-Fraser, 1995) have revealed that segregation of the neural crest

lineage from other neural tube lineages is a relatively late event, occurring around the time of neural crest emigration. Such results demonstrate that neural crest precursors prior to this stage are not committed to either a central nervous system or neural crest fate. Using notochord grafts as a means of challenging the fate of neural crest precursors, Artinger and Bronner-Fraser (1992, 1995) found that notochords overlying the closed neural tube were unable to suppress the formation of neural crest cells; they could, however, induce the formation of floor plate cells and motor neurons. Such results suggest that at this stage, neural crest precursors have been induced such that they cannot be prevented from forming neural crest cells by the presence of a strong ventralizing signal. In contrast, Dickinson et al. (1995) found that the notochord is able to antagonize induction of *Wnt-1* and *Wnt-3a* by the ectoderm, and Liem et al. (1995) have shown that the notochord can suppress expression of the neural crest markers *Slug* and HNK-1 in vitro. In both cases, however, the responding neural tissue was taken from levels of the neuraxis where the neural plate still lies open, i.e. prior to neural tube closure.

This raises the intriguing possibility that neural crest cell specification occurs at a time prior to neural tube closure and can be prevented by grafting notochords at these earlier stages of development. To address this, we implanted notochords adjacent to the neural folds at the open neural plate stage. We find that notochords implanted at this time are indeed able to prevent the formation of migratory neural crest cells. The notochord expresses Shh, and inhibitors of BMP-signaling such as Noggin (Connolly et al., 1997; Marcelle et al., 1997) and Chordin (Streit et al., 1998). To test the function of individual molecular constituents of the notochord, cells expressing Shh or Noggin were implanted adjacent to neural folds at open neural plate stages or adjacent to the closed neural tube. Shh mimicked the effects of the notochord since Shh-expressing cells were able to prevent neural crest emigration only if implanted at open neural plate stages. In contrast, Noggin-secreting cells down-regulated *Slug* expression and prevented neural crest emigration around the time of neural tube closure. Similarly, when added to neural folds in culture, Shh inhibited neural crest production at early times whereas Noggin exerted its effect later. Together, our results suggest three pre-migratory phases of neural crest formation – an early BMP-independent (Shh-sensitive) phase, a later BMP-dependent phase mediated by BMPs in the dorsal neural tube and a third, Shh- and Noggin-insensitive, phase just prior to neural crest emigration.

MATERIALS AND METHODS

Notochord implants

The trunk regions of stage 10-12 chick embryos (Hamburger and Hamilton, 1951) were isolated using iridectomy scissors, and incubated in 160 units/ml of collagenase (Worthington Biochemical) for 15 minutes on ice, followed by 8 minutes at 37°C. The trunk tissues were separated by gentle trituration with a heat-pulled Pasteur pipette, the notochords were isolated and allowed to recover from their enzymatic isolation in Modified Eagle's Medium (MEM) with 15% horse serum and 10% embryo extract for 30-90 minutes on ice prior to implantation.

Embryos at stages 9-14 were windowed as described previously (Stern, 1993; Selleck, 1996). Using tungsten needles, the vitelline membrane overlying the caudal neural tube/open neural plate was deflected. In some experiments, neural fold cells or dorsal neural tube cells were labeled with DiI as described below. Unilateral or bilateral incisions were made through the ectoderm immediately lateral to the neural folds or neural tube. Notochords were guided through the cuts and aligned rostrocaudally beneath the neural folds or adjacent to the dorsal neural tube. Eggs were subsequently sealed with electrical tape and incubated for 9-36 hours at 38°C.

Implantation of Sonic Hedgehog- and Noggin-secreting cells

Cells expressing Shh were generated by transfecting primary fibroblasts obtained from line O chick embryos (Morgan and Fekete, 1996) with a Shh retroviral construct (a gift from Dr Cliff Tabin). Cells were harvested for use at least one week after transfection. CHO cells expressing Noggin, and the dhfr-CHO control cells, were a kind gift from Dr. Richard Harland.

Prior to injections, cells were removed from the tissue culture vessel by treatment with trypsin-EDTA (Gibco-BRL), and the cells allowed to recover for 60 minutes in culture medium containing 20% fetal calf serum. After centrifugation, cells were resuspended in DiI solution (1 mg/ml in ethanol; Molecular Probes), or CellTracker Green CMFDA (1 mg/ml in ethanol; Molecular Probes) in Howard Ringer's (HR) saline and incubated at 38°C for 15 minutes. Following a brief wash, cells were centrifuged and resuspended in a small volume of Howard Ringer saline.

Labeled cells were back-loaded into glass micropipettes, which were then attached to a General Valve Picospritzer. The tips of the micropipettes were broken with forceps to generate an opening through which the cells could pass. Embryos at stages 8-13 were prepared as described above and the vitelline membrane deflected. When cells were injected at open neural plate levels (around stage 10), a small incision was made lateral to the neural folds, as for notochord grafting experiments. The pipette tip was lowered through this opening and cells were injected beneath the neural folds. After further incubation, the injected cells were frequently found adjacent to more ventral regions of the neural tube, suggesting that folding of the neural plate resulted in displacement of these cells relative to the neural folds and dorsal neural tube. Injections at more rostral levels of stage 12-13 embryos were made either into the lumen of the closed or closing neural tube, or outside the neural tube, between the tube, epidermis and paraxial mesoderm. Optimal results were obtained when the cell suspension was of such consistency that cells could be extruded from the pipette as a firm aggregate. Injected embryos were re-incubated for a further 6-30 hours.

DiI injections

CellTracker CM-DiI (C-7000; Molecular Probes) was dissolved in absolute ethanol to a concentration of 1 mg/ml and further diluted in 9 parts of 10% sucrose in water just before starting the injections. Glass micropipettes were back-filled with the DiI solution and attached to a General Valve Corporation Picospritzer II assembly. After windowing the eggs and tearing the vitelline membrane (see above), a DiI-filled micropipette was lowered onto the embryo until its tip lay in contact with the neuroepithelium. In some experiments, focal injections of DiI were made into neural folds or dorsal neural tube cells. In other experiments, more extensive regions of neural tube were labeled by injecting DiI into the lumen of the neural tube.

Whole mount in situ hybridization

Digoxigenin-labeled riboprobes were synthesized in vitro using standard protocols (Nieto et al., 1996). The following probes were used in our experiments: *Slug* (Nieto et al., 1994), BMP-4 (Duprez et al., 1996), *Pax-3* (Stark et al., 1997), *Wnt*-1 (Hollyday et al., 1995).

Whole mount in situ hybridizations were performed as described previously (Henrique et al., 1995). Embryos were fixed once the color reaction had reached completion and photographed in whole mount.

Some of the embryos were subsequently embedded in wax and examined in transverse sections.

Fixation and sectioning

Processed embryos were dehydrated through an ethanol series, cleared in Histosol and embedded in Paraplast. DiI-labeled embryos were lightly stained in fast green to render them visible in the paraffin blocks. 12 μm sections were cut on a Leitz microtome and mounted onto slides covered with 0.2% gelatin in water. After drying, sections were dewaxed in three changes of histosol and coverslipped in Permount. Fast green staining was removed from sections by rehydrating them through an ethanol series and washing them extensively in PBS, before mounting in Gel/Mount (BioMeda).

Treatment of cultured neural folds with Shh or Noggin

Neural fold fragments, 100 to 200 μm in length, were isolated from the caudal (open neural plate) region of stage 8-10 embryos. Dissections were performed in a solution of trypsin (0.1% in calcium- and magnesium-free Tyrode's saline) to ensure complete removal of mesoderm from the ectoderm fragments. The neural fold isolates were transferred to Modified Eagle's Medium (MEM) with 15% horse serum and 10% embryo extract for 30 minutes on ice prior to embedding in collagen gels.

Collagen matrix gel was prepared following previously described protocols (Tessier-Lavigne et al., 1987; Artinger and Bronner-Fraser, 1993). 90 μl collagen (Collaborative Research) was vortexed with 10 μl of 10× DMEM and approximately 5.0 μl 7.5% $NaHCO_3$. 10 μl of the collagen matrix solution was plated onto the bottom of a tissue culture dish and allowed to gel for about 20 minutes. Neural folds were transferred to the surface of this collagen cushion and covered with 3 μl of the gel solution. After a further 20 minutes, collagen gels were submerged in Ham's F12 (Gibco-BRL) containing N2 supplement and penicillin/streptomycin. At various times after the start of culture, Shh (a gift from Eric Turner) or Noggin (*Xenopus*; a gift from Richard Harland) protein were added to the medium at concentrations of 40 μg/ml or 50 ng/ml respectively. Shh was used at this concentration because our preliminary experiments showed that this concentration was able to completely inhibit *Slug* expression in cultured neural folds. Noggin was used at 50 ng/ml because this concentration is effective in dorsalizing Xenopus mesoderm (Lamb et al., 1993) and it inhibits BMP-2, -4 and –7 (Zimmerman et al., 1996).

After 5 hours of incubation in the protein, the collagen gel explants were rinsed three times in fresh F12+N2 and cultured for the remaining time in this defined medium. Tissues were incubated at 38°C in a gassed tissue culture incubator for a total of 48 hours, at which time they were fixed in 4% paraformaldehyde for 1 hour at room temperature and processed for HNK-1 antibody staining.

Whole-mount HNK-1 antibody staining

Whole-mount antibody staining was performed as described previously (Selleck and Stern, 1992). After fixation, collagen gels were rinsed several times in PBS and PBT (PBS containing 0.2% BSA and 1% Triton X-100) and incubated in HNK-1 hybridoma supernatant overnight at 4°C. The following day, specimens were washed extensively in PBT and incubated in HRP-conjugated goat anti-mouse IgM secondary antibody overnight. After more washing in PBS and 0.1 M Tris (containing 100 mM NaCl, at pH 7.4) HRP activity was revealed by immersing the collagen gels in 30 mg/ml DAB to which hydrogen peroxide was added to a final concentration of 0.001%.

Examination of embryos

Fluorescently labeled embryos were examined in whole mount and in section by epifluorescence microscopy and photographed using Kodak Ektachrome 400 slide film. A subset of these embryos was selected for examination by confocal microscopy. In situ hybridizations and HNK-1-labeled neural fold fragments were photographed using Kodak Ektachrome 160T slide film. Color slides were subsequently imported into Adobe Photoshop using a Kodak SprintScan slide scanner. Digitized confocal images were also imported into Adobe Photoshop. Composite images were manipulated to enhance visualization of the fluorescent label and printed on a Kodak XLS color printer.

RESULTS

Notochords implanted into the open neural plate suppress neural crest migration

Notochords grafted dorsal to the neural tube can induce the formation of ectopic floor plates and motor neurons (Yamada et al., 1991), but are unable to suppress the formation of neural crest cells (Artinger and Bronner-Fraser, 1992). One possible explanation for these results is that some dorsal neural tube cells may be committed to a neural crest precursor cell fate after neural tube closure. To elucidate the time at which such specification occurs, we have grafted notochords at earlier stages of development (designated as 'early grafts'), beneath the neural folds in the open neural plate of stage 9-11 embryos. In most experiments, a longitudinal incision was made through the non-neural ectoderm lateral to the neural folds to permit introduction of the notochord grafts. When this operation was performed without subsequently implanting notochords, neural crest emigration and *Slug* expression proceeded normally (data not shown).

To examine the effects of notochord implants on neural crest emigration from the neural tube, we followed the fate of adjacent neural fold cells by labeling them with the fluorescent marker, DiI. Since the notochord is known to inhibit neural crest migration (Pettway et al., 1990), we grafted notochords unilaterally and DiI-labeled the ipsilateral neural fold cells. In the event that neural crest cells were generated from the neural folds, but were inhibited from migrating by the grafted notochord, the absence of a notochord on the contralateral side should permit those cells to cross the midline and migrate normally. Engrafted embryos were incubated for a further 15-24 hours to stages 14-15.

We performed 21 unilateral grafting and DiI-labeling experiments. Despite verification that the initial DiI injection was limited to those cells immediately adjacent to grafts, we found after further incubation that DiI label in the neural primordium extended beyond the rostrocaudal levels of the notochord graft. Thus, we observed neural crest cells migrating bilaterally from the neural tube both rostral and caudal to the grafted notochord, in keeping with results from unoperated control embryos (n=5; Fig. 1A). In contrast, a region of neural tube lying within two somite-diameters of the notochord graft failed to generate migratory cells (n=14; Fig. 1B-D). As a control, notochords were fixed in paraformaldehyde and washed extensively prior to grafting (n=4). In all cases, segmental, bilateral streams of neural crest cells could be observed migrating from the neural folds adjacent to implanted notochords, in one case migrating over the graft itself (Fig. 1E,F).

The developmental properties of this same cell population were examined at a later developmental stage (designated as 'late grafts') by grafting notochords dorsolateral to the closed neural tube of stage 12-13 embryos, i.e. at the level of rostral

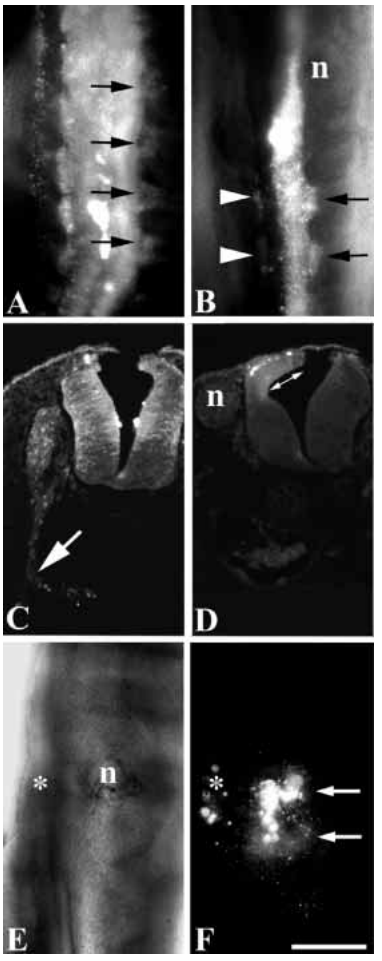

Fig. 1. DiI labeling of neural fold or neural tube cells reveals that an implanted notochord can suppress migration of the adjacent neural crest. (A) An unoperated embryo viewed from the dorsal side after DiI-labeling of the neural tube. DiI-labeled neural crest cells were seen migrating as segmental streams (arrows) from both sides of the neural tube. (B) An embryo viewed from the dorsal side after unilateral DiI-labeling of the neural fold followed by a notochord graft (demarcated by 'n'). Adjacent to the notochord implant, no neural crest cells emerged from the folds. In contrast, caudal to the notochord implant, neural crest cells emigrated in segmental streams both ipsilateral (arrows) and contralateral (arrowheads) to the labeled neural folds. (C,D) Transverse sections through another embryo with a notochord graft and DiI-labelled neural folds. (C) In a section caudal to the graft, labeled neural crest cells (arrow) emigrated from the dorsal neural tube and populated the forming sensory and sympathetic ganglia as well as contributing to Schwann cells. (D) Adjacent to the implanted notochord, DiI-labeled neural fold cells (double-headed arrow) failed to generate migratory neural crest cells either unilaterally or bilaterally. (E,F) When a fixed notochord was implanted adjacent to the neural folds, neural crest cells were not prevented from forming or emigrating. (E) Whole mount view of the implanted fixed notochord (n) indicating its position immediately lateral to the dorsal neural tube (asterisk). (F) DiI-labeled neural crest cells emerging from the focally labeled neural folds emigrated from the dorsal neural tube in two streams (arrows), with some migrating on the implanted notochord itself. Scale bar, 100 μm.

segmental plate (*n*=23). In contrast to notochord implants at open neural plate levels, notochords grafted adjacent to the rostral segmental plate were unable to perturb neural crest migration (*n*=18; data not shown), confirming our previous studies (Artinger and Bronner-Fraser, 1992, 1995).

Notochord implanted adjacent to the open neural plate suppress *Slug* expression

In addition to analyzing neural crest emigration from the tube using DiI-labeling, we examined expression of the zinc finger transcription factor *Slug* as a marker for neural crest cells after 'early grafts' of notochords adjacent to the open neural plate. *Slug* is the earliest known avian neural crest marker, being expressed in the dorsal neural tube prior to neural crest emigration and subsequently in early migrating neural crest cells (Nieto et al., 1994). Embryos were observed 15-24 hours post-grafting, by which time the neural tube had closed and the first neural crest cells were beginning to migrate. Along the length of the neural tube that lay adjacent to the grafted notochord, we observed a complete elimination of *Slug* expression (Fig. 2). The results were consistent in all embryos examined (*n*=12). Both unilateral and bilateral notochord grafts prevented *Slug* expression: unilateral grafts inhibited *Slug* expression unilaterally, whereas bilateral grafts completely eliminated *Slug* in the grafted region. Moreover, notochords lying at some distance from the dorsal neural tube (approximately 50 μm) were still able to perturb *Slug* expression in neural fold cells. In contrast to grafted regions, strong *Slug* expression was observed in the neural tube both rostral and caudal to the site of notochord implantation. These results suggest that prior to neural tube closure, repressive signals from the notochord are able to antagonize induction of *Slug*.

We also performed 'late grafts' in which notochords were implanted at the level of the rostral segmental plate of stage 12-13 embryos (*n*=18). While we have shown that such grafts are unable to prevent neural crest emigration from the adjacent neural tube, we found that such notochord implants retained the ability to down-regulate *Slug* expression at this later developmental stage.

We examined the effect of the notochord on expression of BMP-4, a candidate for the dorsalizing effects of non-neural ectoderm (Liem et al., 1995). At open neural plate regions, BMP-4 is expressed at low levels in the superficial ectoderm lateral to the neural folds, and at high levels in the neural folds themselves. As development proceeds, BMP-4 expression remains within the neural folds and dorsal neural tube, and disappears from the prospective epidermis (Watanabe and Le Douarin, 1996). Notochords were implanted unilaterally or bilaterally beneath the open neural plate and operated embryos were incubated for a further 9-18 hours, fixed and processed for in situ hybridization (*n*=11). In the majority of embryos (7/11; 2 unilateral, 5 bilateral), early BMP-4 expression in the dorsal neural tube/neural folds was unaffected by the presence of the notochord(s), even when the notochords lay in direct contact with the neuroectoderm (Fig. 2G,H). In these specimens, the grafted notochords lay adjacent to the caudal segmental plate (corresponding to an incubation time of 9-12 hours). In 2/11 embryos (1 unilateral graft; 1 bilateral graft), BMP-4 expression in the neural tube was down-regulated (Fig. 2I). In these 2 embryos, the notochords lay adjacent to the rostral end of the segmental plate (15-18 hours of incubation). We conclude that the ability of the notochord to down-regulate

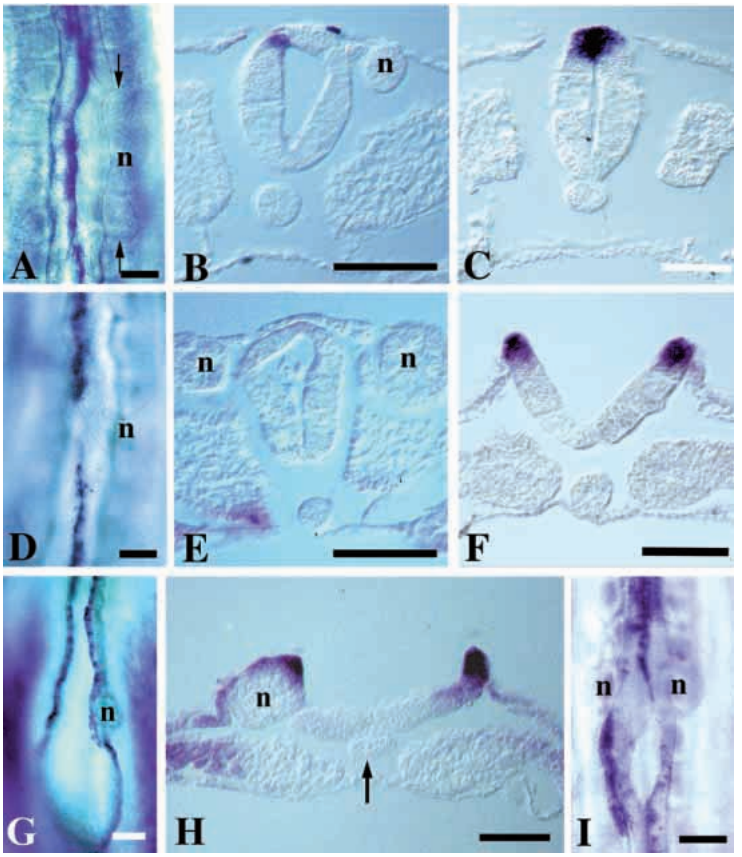

Fig. 2. A notochord implanted beneath the open neural folds can suppress the expression of *Slug*, but only down-regulates BMP-4 expression after prolonged contact. (A) Following a unilateral notochord graft (n and arrows), *Slug* expression was eliminated in the ipsilateral neural tube, while rostral and caudal to the graft site, *Slug* is expressed bilaterally. (B) In transverse section, the grafted notochord (n) induced wedging in the adjacent neural tube cells, indicative of an induced floor plate. In this case, *Slug* expression was reduced to a small group of dorsal neural tube cells on the contralateral side. (C) Transverse section through a control embryo probed with *Slug* reveals that expression extends across the dorsal-most aspect of the neural tube. (D,E) After bilateral implantation of notochords ('n') beneath the neural folds, *Slug* expression was completely suppressed in the adjacent stretch of neural tube, but remained unaffected rostral and caudal to the notochord implant. (F) BMP-4 is expressed at high levels in neural fold cells at open neural plate stages, and at much lower levels in the adjacent non-neural ectoderm. Following neural tube closure, BMP-4 expression was maintained in the dorsal neural tube, but absent from the ectoderm. Unilateral (G,H) notochord implants failed to down-regulate BMP-4 expression 9-12 hours after surgery, despite close contact between the notochord implant (n) and the adjacent neural folds. In these specimens, complete closure of the neural tube was prevented by the engrafted notochords. (I) Another embryo, 15 hours after bilateral notochord implants, exhibits some down-regulation of BMP-4 expression adjacent to the notochords. Scale bars, 100 μm.

BMP-4 expression correlates with the incubation time of the host embryos. Two possible explanations for these results are that: (i) repression of BMP-4 by the notochord may not be an immediate response, but may increase with time of contact, or (ii) BMP-4 expression becomes sensitive to signals from the notochord only after neural tube closure. This subsequently led us to perform tissue culture experiments, which confirm that timing is critical for inhibition of neural crest formation (see Fig. 7).

Sonic Hedgehog-producing cells grafted into the open neural plate suppress neural crest emigration

What molecules present in the notochord might account for its ability to inhibit neural crest emigration and *Slug* expression? Shh is a molecule expressed in the notochord that has been shown to pattern the developing neural tube by inducing the

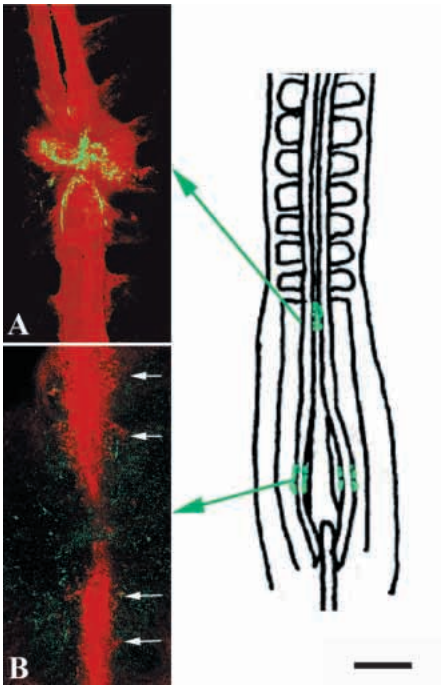

Fig. 3. Shh-expressing cells suppress neural crest migration if injected into the open (B) neural plate, but not if injected adjacent to the closed (A) neural tube. (A) This confocal micrograph shows the result of a luminal injection of Shh-expressing cells into the closed neural tube, at the levels indicated on the schematic. The neural tube was labeled with DiI (red) prior to injection of the cells (yellow-green). After further development, segmental streams of DiI-labeled neural crest cells were seen migrating away from the neural tube, including regions of the neural tube adjacent to the implanted cells. (B) Bilateral injections of Shh-expressing cells beneath the neural folds severely perturbed subsequent development of the neural tube and inhibited neural crest migration. This confocal micrograph shows an optical section through the ventral-half of the neural tube and segmental streams of neural crest cells were discerned both rostral and caudal to the cell injection site (arrows). At the site of injection, no neural crest cells were seen migrating through the mesoderm. Because of distortion of the neural tube in the vicinity of the cell implant, only a small portion of the affected neural tube was obvious in this optical section. Scale bar, 100 μm.

formation of floor plate cells and motor neurons. One possibility, therefore, is that Shh within the notochord inhibits neural crest formation by converting prospective neural crest precursors to ventral neural tube derivatives. Shh-expressing fibroblasts were injected unilaterally or bilaterally underneath the neural folds at the open neural plate stage ('early grafts'), or adjacent to or within the closed neural tube at the level of the rostral segmental plate ('late grafts'). Both grafts were performed at the same axial position but were temporally separated by approximately 15 hours. After Shh-injection, the adjacent neural tube often appeared hypertrophied, with occasional elimination of the neural tube lumen. Neural tube hypertrophy was somewhat less severe in late than early grafts. Somites around the injection site were often affected, expanding mediolaterally by about 50-100%. We assayed for the generation of neural crest cells by DiI labeling of neural crest precursors, or analysis of *Slug* expression in the dorsal neural tube. Effects on the dorsal neural tube were assessed by examining BMP-4, *Wnt-1* and *Pax-3* expression.

Analysis of DiI-labeled neural crest cells after 'early grafts' (n=23) revealed an absence of neural crest cells in the paraxial mesoderm adjacent to the injection site, whereas neural crest cells rostral and caudal to the Shh-producing cells were migrating in a normal segmental pattern through the somites (Fig. 3B). The neural crest-free region often extended 2 somite-lengths rostral and caudal to the injection site. This suggests that: (i) Shh protein is able to diffuse extensively through the embryo over a distance of approximately 200 μm, and/or (ii) cells of the open neural plate undergo a significant rostrocaudal rearrangement during neural tube closure. Our previous DiI fate mapping experiments provide evidence in favor of the latter possibility (Selleck and Bronner-Fraser, 1995).

'Early grafts' of Shh-producing cells resulted in extensive rostrocaudal inhibition of *Slug* expression when examined 12-19 hours post-injection (n=16). In the majority of cases (n=15), *Slug* transcripts were absent from a length of neural tube adjacent to the injected cells (Fig. 4A). In contrast, control fibroblasts had no effect (6/6). These results demonstrate that Shh alone is sufficient to cause a local elimination of *Slug* expression in the open neural folds, and inhibit neural crest migration in the open neural plate.

In contrast to 'early grafts', 'late grafts' of Shh-cells failed to eliminate neural crest migration. DiI-labeling revealed normal emigration of neural crest cells away from the neural tube (16 of 18 cases; Fig. 3A) adjacent to the injection site, despite changes in the morphology of the somites. However, analysis of *Slug* transcripts revealed that even at these later times, Shh-secreting cells were able to cause a local down-regulation of *Slug* expression (5/5; Fig. 4B). We also injected Shh-expressing cells into the lumen of the closing neural tube (corresponding to a point halfway along the unsegmented paraxial mesoderm, thus representing an 'intermediate' time point). In most cases (16 of 17 embryos), *Slug* expression was inhibited in the vicinity of the cell bolus.

In addition to *Slug*, we examined the effects of exogenous Shh on dorsal neural tube markers, BMP-4, *Wnt-1* and *Pax-3* after 'early' Shh grafts. Despite their radical effect on the morphology of the neural tube, injections of Shh-fibroblasts had no effect on the subsequent expression of BMP-4 in the dorsal neural tube. Even in severe cases where the neural tube

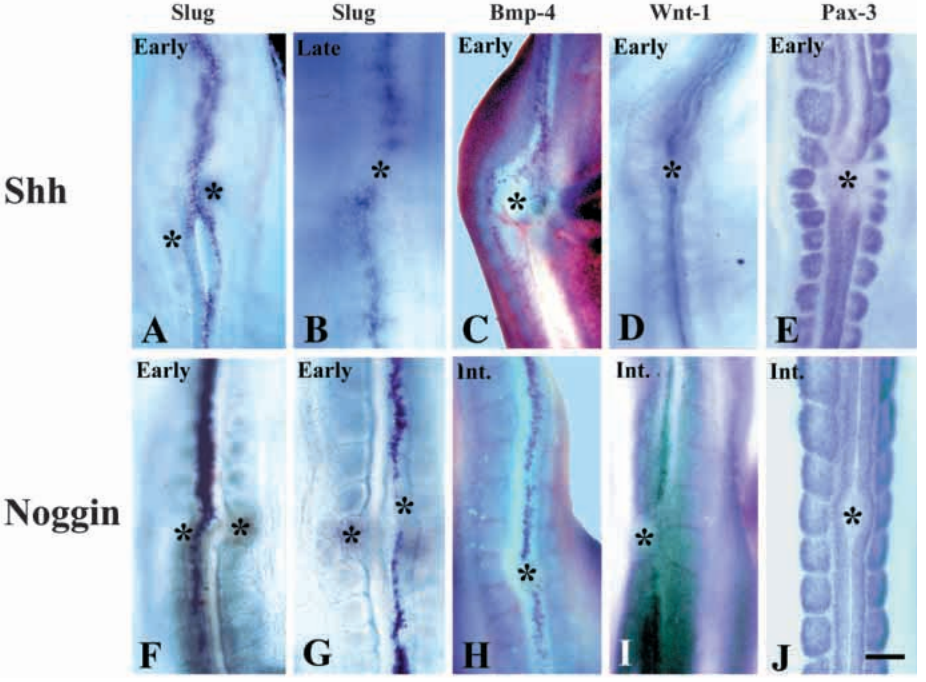

Fig. 4. Shh- and Noggin-expressing cells prevent or down-regulate Slug expression in the dorsal neural tube and influence the expression of other dorsal neural tube genes. (A) Bilateral injections of Shh-expressing cells beneath the neural folds at open neural plate levels ('early' injections) prevent *Slug* expression in the dorsal neural tube. In this specimen, the Shh-cells became displaced ventrally relative to the dorsal neural tube. Nevertheless, *Slug* expression was inhibited in a region of the neural tube that originally lay adjacent to the cell aggregates. Note that the final location of the implanted cells (asterisks) does not correspond to the region of the neural tube lacking *Slug* transcripts. In this specimen, the neural tube failed to close completely – a perturbation that does not, by itself, affect *Slug* expression. (B) Similarly, Shh-expressing cells also down-regulate *Slug* expression when implanted into the lumen of the neural tube at the level of the rostral segmental plate ('late' injections). Shh-expressing cells also influence the expression of some other dorsal neural tube genes if injected at 'early' times. (C) Despite a severe effect on the morphology of the neural tube, Shh-expressing cells failed to suppress expression of BMP-4 in the dorsal neural tube, and expressing cells were seen along the hypertrophied roof of the tube. In contrast, Shh-producing cells inhibited *Wnt-1* (D) and *Pax-3* (E) expression. (F) Noggin-producing cells injected at open neural plate levels failed to prevent *Slug* expression if the cells moved away from the neural folds during neural tube closure. (G) In contrast, if the injected cells remained in contact with the dorsal neural tube after closure, *Slug* expression was inhibited in the vicinity of the implanted cells. Noggin-expressing cells also inhibited BMP-4 (H) and *Wnt-1* (I) expression in the adjacent neural tube, but failed to down-regulate *Pax-3* expression (J). Scale bar, 100 μm.

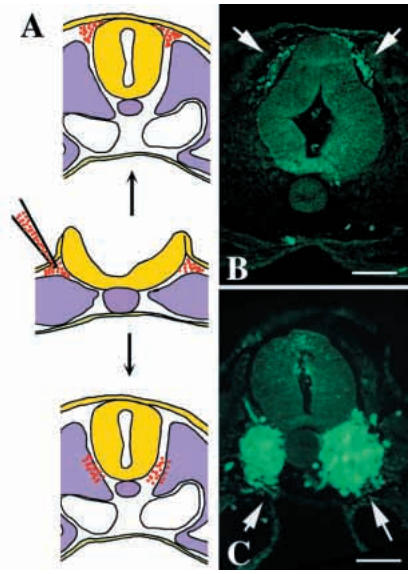

Fig. 5. Cells injected beneath the neural folds at the open neural plate stage frequently become displaced during neural tube closure. (A) A schematic diagram illustrating the behavior of cells (red dots) after injection beneath the neural folds. In some cases, injected cells remained adjacent to the dorsal neural tube after tube closure (top diagram). In the remaining embryos, we found that cells became displaced from the neural folds and came to lie adjacent to the ventral neural tube (bottom diagram). (B) An example of Shh-secreting fibroblasts that had been injected at the open neural plate level of the neuraxis and had subsequently come to lie around the dorsal aspect of the neural tube (arrows). Even though few cells are present at this level, they have induced wedging in the nearby neural tube. (C) A transverse section through an embryo that had received an injection of Noggin-expressing cells reveals that the cells have become displaced ventrally, away from the dorsal neural tube. In this case, the large cell aggregates have come to lie adjacent to the ventral neural tube and notochord. No obvious effects were seen on the adjacent neural tube. Scale bars, 100 μm.

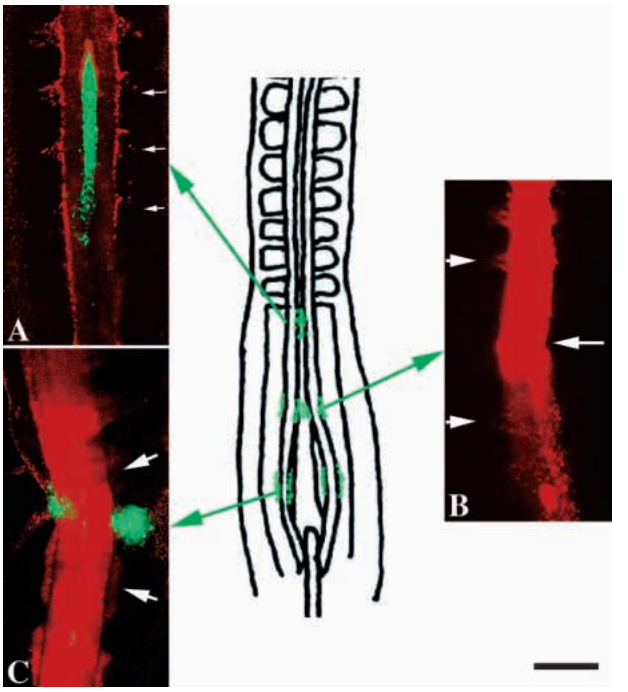

Fig. 6. Noggin-expressing cells suppress neural crest migration if injected into the closing neural tube. (A) Injections of Noggin-expressing cells (green) into the lumen of the neural tube at rostral levels failed to suppress neural crest migration. This confocal micrograph clearly shows segmental streams of DiI-labeled migrating crest cells lateral to the injected cells. (B) In contrast, cells injected around the closing neural tube do suppress neural crest migration (long white arrow), while migration continued unperturbed both rostral and caudal to the injection site (short white arrows). The injected cells are not visible in this optical section. (C) Bilateral injections of Noggin-secreting cells beneath the neural folds at open neural plate levels failed to perturb neural crest migration, and segmental streams were seen emigrating from the neural tube rostral and caudal to the injection site (arrows). Scale bar, 100 μm.

had failed to close correctly, BMP-4-expressing cells could be found along the hypertrophied roof of the gaping neural tube (Fig. 4C). In contrast, Shh-expressing cells did down-regulate expression of both *Wnt-1* (Fig. 4D) and *Pax-3* (Fig. 4E).

These results demonstrate that Shh has different effects on neural crest cell emigration at ‘early’ versus ‘late’ times. The results for all the markers used in this study are summarized in Fig. 8. Early, but not late, injections eliminate neural crest migration. Both early and late injections cause down-regulation of *Slug*. Moreover, ‘early’ Shh injections inhibit *Wnt-1* and *Pax-3* expression, but do not affect BMP-4 expression in the dorsal neural tube.

Noggin-producing cells suppress neural crest emigration and *Slug* expression shortly after tube closure

Both Noggin and another BMP-signaling antagonist, Chordin (Piccolo et al., 1996), are expressed by the notochord (Marcelle et al., 1997; Streit et al, 1998). Because BMP signaling has been implicated in neural crest induction (Liem et al., 1995), it is possible that the effects of the notochord on neural crest formation are mediated by BMP signaling antagonists. To test this possibility, Noggin-expressing cells were implanted in the same manner as described for Shh-cells above. We examined the ability of ‘early grafts’ and ‘late grafts’ of Noggin-producing cells to suppress neural crest formation by examining *Slug* expression and by DiI labeling the neural folds or neural tube. We have also analyzed the expression of the dorsal neural tube markers BMP-4, *Wnt-1* and *Pax-3* after grafts of Noggin-expressing cells.

The effects of ‘early grafts’ on *Slug* expression appeared to depend on the distribution of the injected cells around the neural tube after its closure. We often observed that Noggin-expressing cells became displaced away from the neural folds during neural tube closure (Fig. 5) and found in such cases that *Slug* expression was largely unaffected (Fig. 4F; n=10). In contrast, when the cells remained closely associated with the neural folds and consequently came to lie around the dorsal neural tube, *Slug* expression was completely abolished (Fig. 4G; n=7). Control cells had no effect in any surviving embryos (n=15). Two possibilities could account for these differences. One possibility is that prolonged contact is required between the noggin-expressing cells and the neural folds to prevent *Slug* expression. The ventral displacement of cells that was often observed during

neural tube closure would therefore limit the exposure of neural fold cells to Noggin (to an estimated 5 hours). We argue against this possibility because we found in preliminary experiments that Noggin-expressing cells are able to down-regulate *Slug* expression within just 2-3 hours. Another, more likely, possibility is that *Slug* expression is sensitive to Noggin later, around the time of neural tube closure. Ventral displacement of secreting cells would therefore remove Noggin from the neural folds before the time at which *Slug* expression becomes dependent on BMP signals. The pattern of neural crest migration, as assessed by DiI-labeling, was unaltered in the presence of exogenous Noggin introduced at early stages (Fig. 6C).

For 'late grafts', Noggin-expressing cells were injected into the lumen of the neural tube adjacent to the rostral segmental plate. In all cases, *Slug* expression in the vicinity of the cells was down-regulated (n=20), while CHO control cells showed no effect (n=18). When DiI was used to label the adjacent neural fold cells, we observed normal neural crest emigration in most specimens (9/10; Fig. 6A). Interestingly, injections of Noggin-expressing cells into, or adjacent to, the closing neural tube at intermediate levels resulted in an inhibition of neural crest cell migration (11/13; Fig. 6B).

Noggin-expressing cells had differential effects on expression of transcripts expressed in the dorsal neural tube. Noggin down-regulated expression of BMP-4 in the dorsal neural tube (Fig. 4H), suggesting that BMP-4 expression is dependent on levels of its own protein. Like Shh-expressing cells, Noggin also suppressed expression of *Wnt-1* (Fig. 4I), but had no effect on *Pax-3* expression (Fig. 4J).

These results demonstrate that Noggin has temporally distinct effects on formation of the neural crest. The results for all the markers used in this study are summarized in Fig. 8. Noggin altered the pattern of neural crest migration when injected into the closing neural tube. Late, but not early, Noggin injections down-regulated *Slug* expression, in contrast to the effect observed with Shh. Intermediate Noggin injections cause down-regulation of BMP-4 and *Wnt-1* but had not effect on *Pax-3* . Hence, the ability of Noggin to influence neural crest formation is stage dependent, with no apparent effect in the open neural plate (when BMP is expressed in the ectoderm), but a significant effect at the time of neural tube closure (when BMP is expressed in the neural folds/dorsal neural tube).

Timing of neural crest formation in vitro

The above experiments suggest differential and stage-dependent effects for both Shh and Noggin on neural crest formation. Whereas Shh-expressing cells inhibit neural crest formation and migration at open neural plate stages, but not after neural tube closure, Noggin-expressing cells appear to prevent neural crest formation only later, around the time of neural tube closure.

To refine our analysis of the stage-dependent influences of Shh and Noggin on neural crest formation, we examined Shh- and Noggin-sensitive periods of neural crest development in a defined culture system. Isolated neural folds from the open neural plate region of stage 8-10 embryos were grown in collagen gels in the presence of either Shh or Noggin protein. The initial 15 hour culture time was divided into three 5-hour periods, treatment with protein being limited to any one of the three periods, followed by further culture in defined medium for a total of 48 hours. In most cases, we assayed the ability of the neural folds to produce neural crest cells by their HNK-1 immunoreactivity rather than *Slug* expression, because it is apparent from our in vivo experiments that inhibition of *Slug* expression does not necessarily correlate with a suppression of neural crest emigration. Therefore, HNK-1 is a better indicator of neural crest formation than *Slug* expression in this assay.

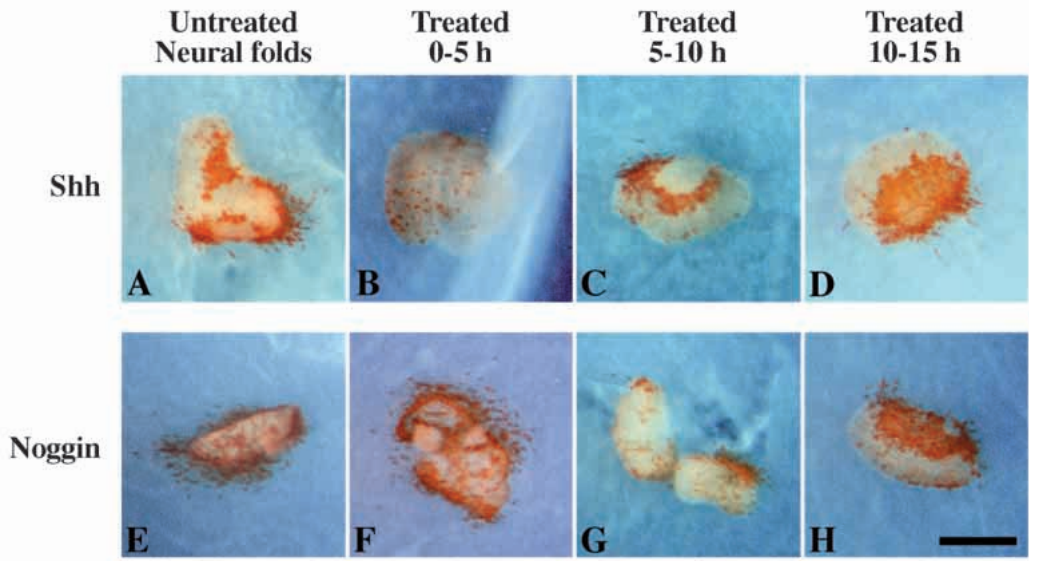

Fig. 7. In vitro addition of Shh-expressing cells (A-D) and Noggin-expressing cells (E-F) to neural fold isolates confirms the existence of different periods of sensitivity to each protein. (A,E) Neural folds cultured in defined medium for 48 hours are intensely HNK-1-positive and numerous labeled cells were observed migrating away from the neural fold explant. (B) Treatment of neural fold isolates with Shh protein within the first 5 hours of culture significantly diminished HNK-1 staining, leaving only a small number of punctate, HNK-1 immunoreactive cells in the neural fold. No positive cells were seen migrating from the neural fold explant. In contrast, treatment of neural folds with Shh between 5 to 10 hours (C) or 10 to 15 hours (D) of culture had little effect on HNK-1 immunostaining, and such explants resembled the controls shown in A and E. (F) If treated with Noggin protein at 0-5 hours of culture, neural folds continued to produce numerous HNK-1-positive cells, many of which were located outside the explant, migrating through the collagen gel matrix. In contrast, (G) treatment with Noggin between 5-10 hours of culture significantly reduced, but did not eliminate, HNK-1 expression in the explants. (H) Treatment with Noggin between 10 and 15 hours of culture had little effect on HNK-1 immunoreactivity since the intensity of HNK-1 staining was comparable to that of the controls (E). Scale bar, 100 μm.

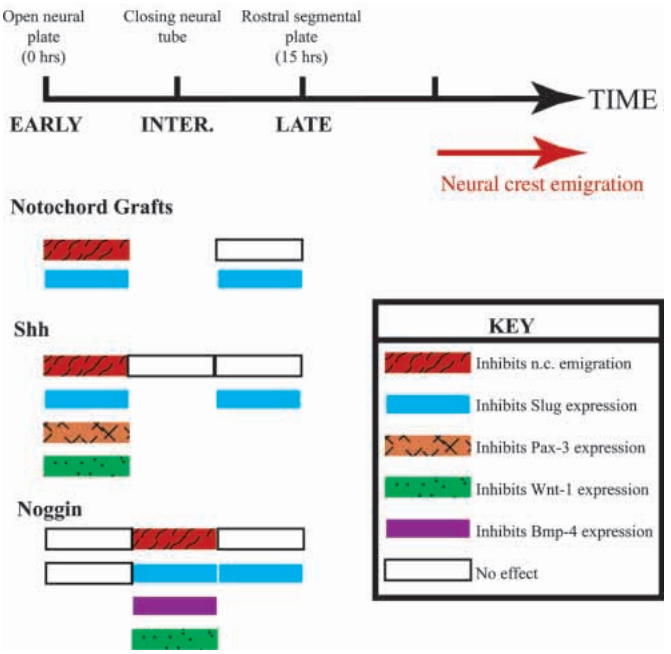

Fig. 8. Summary of data on the temporal effects of the notochord, Shh and Noggin and neural crest emigration and expression of *Slug, Wnt-1, Pax-3* and BMP-4. A time line of neurulation is presented at the top. Below are the times during which each type of graft effects each marker.

Control neural folds cultured for a total of 48 hours in defined medium contained numerous HNK-1-positive cells both within and migrating away from the explant (Fig. 7A,E). Treatment of neural fold explants with Shh for the entire 48 hour culture period completely eliminated the migratory cell population, and significantly reduced the presence of HNK-1 immunoreactivity within the explant. The small, punctate immunopositive cells within the explant had a very different appearance compared with the premigratory positive cells in untreated neural folds, suggesting that they are not neural crest precursors (data not shown). When neural folds were treated with Shh for the first 5 hours of culture, and cultured for the remaining time in defined medium, formation of migratory HNK-1-positive cells was similarly prevented and the HNK-1-positive cells within the explant were small and punctate (Fig. 7B). In contrast, neural folds exposed to Shh protein between 5 and 10 hours of culture (Fig. 7C), or 10 and 15 hours (Fig. 7D) gave rise to migratory HNK-1-positive cells and intense immunoreactivity within the explant itself, similar to untreated controls. Together, these results demonstrate that generation of neural crest cells from isolated neural folds can be perturbed by Shh during the first 5 hours of culture only, which corresponds in vivo to the open neural plate stage of ('early') neurogenesis.

A reduction in HNK-1 immunoreactivity was also seen after treating neural plates with Noggin. However, the reduction in formation of neural crest cells coincided with the 5-10 hour treatment period (Fig. 7G), with no observable effects when treated between 0-5 (Fig. 7F) and 10-15 (Fig. 7H) hours of culture. The Noggin-mediated reduction in HNK-1 immunoreactivity was milder than that observed with Shh, with a few migratory HNK-1+ cells continuing to form. Interestingly, neural folds cultured for the entire 48 hours in Noggin showed no reduction in HNK-1 immunoreactivity. This may indicate that the Noggin protein is unstable and does not remain in active form for longer than a few hours in our culture system. Taken together, our data provide strong evidence that neural folds become sensitive to Noggin between 5 and 10 hours of culture, corresponding in vivo to mid-segmental plate ('intermediate') levels of neurogenesis, i.e. around the time of neural tube closure.

In a series of experiments, we assayed Shh and Noggin proteins for their ability to suppress *Slug* expression. Explants were cultured in the presence of either protein for 18 hours, after which the gels were fixed and hybridized to a *Slug* probe. Neural folds cultured in the presence of Shh (40 μg/ml) failed to express detectable levels of *Slug*, whereas untreated controls displayed prominent *Slug* expression (data not shown). In contrast, Noggin protein (50ng/ml) failed to suppress *Slug* expression – either in neural folds or in neural plate/epidermis recombinants (data not shown). Noggin protein, when presented at the beginning of the culture period, may be rapidly inactivated or degraded in our culture system and, therefore, is not present in sufficient quantities at the 'sensitive period' to affect *Slug* expression.

Together, these culture experiments confirm our previous in vivo data and show that neural crest formation has temporally distinct periods of sensitivity to Shh and Noggin. Whereas Shh can prevent neural crest formation at early, open neural plate stages, Noggin has no effect at this time-point. Conversely, Noggin inhibits neural crest formation around the time of neural tube closure, when Shh has little effect on emigration of neural crest cells.

DISCUSSION

The dorsoventral polarity of the neural tube is thought to be established by interactions with adjacent non-neural tissues. To date, the notochord and the prospective epidermis have been shown to possess polarizing ability. The notochord, via a Sonic hedgehog signal (Echelard et al., 1993; Roelink et al., 1995), can induce adjacent neural tube cells to develop into ventral derivatives such as motor neurons and floor plate (van Straaten, 1988; Yamada et al., 1991). The superficial, non-neural ectoderm can induce neural crest, a dorsal derivative (Moury and Jacobson, 1990; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995), and both BMP-4 and BMP-7 have been shown to be able to substitute for the ectoderm in inducing neural crest cells (Liem et al., 1995). We report here the result of experiments, designed to examine the timing of neural crest cell precursor commitment, using ventralizing agents or inhibitors of BMP-signaling to challenge the fate of prospective neural crest cells at different times of development. We injected Shh- or Noggin-expressing cells, or grafted notochord fragments, at the open neural plate level of stage 10 embryos ('early grafts'). To target this same cell population at a later stage, we implanted cells or notochords at the level of the rostral segmental plate in embryos at stages 12-13 ('late grafts'). We find that the notochord and Shh are able to inhibit neural crest formation at open neural plate stages, whereas Noggin does so later, around the time of neural tube closure. The data from our experiments are summarized schematically in Fig. 8.

Notochords and Shh-secreting cells suppress neural crest formation at open neural plate stages

Our results show that notochords grafted adjacent to the neural folds at open neural plate stages suppress neural crest formation and emigration, assayed by *Slug* expression and DiI labeling of neural crest precursors respectively. The products of a number of genes expressed in the notochord could account for its ability to inhibit neural crest emigration. One possibility is that Shh from implanted notochords ventralizes uncommitted neuroepithelial cells and prevents them from subsequently responding to dorsalizing signals.

By grafting Shh-producing cells adjacent to neural folds at open neural plate stages of development, we have determined that Shh mimics the effects of grafted notochords by completely down-regulating the dorsal marker *Slug* and suppressing the formation of migratory neural crest cells. Both the notochord and Shh data therefore suggest that the neural crest is not determined at open neural plate stages of development.

The time at which neural crest formation is sensitive to Shh appears to be limited to open neural plate levels (equivalent to caudal segmental plate levels in older embryos). First, we found in many cases that cells injected beneath open neural folds remained in contact with them for an estimated 4-6 hours, after which folding of the neural plate caused them to become displaced away from the neural crest precursors. Nevertheless, such limited contact resulted in an inhibition of both *Slug* expression and neural crest emigration. Second, injection of Shh-expressing cells into the neural tube at rostral segmental plate levels failed to inhibit neural crest cell migration. Third, our in vitro experiments suggest that Shh can suppress neural crest formation in neural fold isolates only if applied during the first 5 hours of culture, but has little effect if added to cultures at later times.

This period of sensitivity to Shh coincides with the time at which ventral neural tube cell types are likely to be induced. Artinger and Bronner-Fraser (1993) found that cultured neural plates, isolated from ectoderm immediately rostral to Hensen's node, were able to generate autonomously both floor plate cells and motor neurons, indicating that they had been induced by this early stage. It is even conceivable that the floor plate cells are induced by chordamesoderm precursors while still in Hensen's node ectoderm (Selleck and Stern, 1991; see also Catala et al., 1996). Therefore, the most likely explanation for the activity of Shh is that it ventralizes early neural plate cells, preventing them from responding to subsequent dorsalizing signals from the epidermis, or from BMP-mediated signals from the dorsal neural tube itself. Interestingly, Shh is unable to effect an efficient down-regulation of BMP-4, indicating either (i) that Shh-inhibition acts downstream of BMP-4, or (ii) that two distinct signaling pathways are required for neural crest formation, one Shh-sensitive and the other mediated by BMPs.

The timing of BMP-4 induction of neural crest cells

Noggin is a secreted protein which is expressed in the organizer of frog and mice and which appears to play an important role in neural induction (Lamb et al., 1993). Recently, Noggin has been shown to function at a biochemical level by binding to the BMPs, primarily BMP-2, BMP-4 and to a lesser extent BMP-7 (Zimmerman et al., 1996; Holley et al., 1996), thus preventing them from binding to their receptors. Since Noggin and another BMP-signaling antagonist, Chordin (Piccolo et al., 1996), are expressed by the notochord (Connolly et al., 1997; Marcelle et al., 1997; Streit et al, 1998), and both BMP-4 and BMP-7 have been implicated in neural crest induction (Liem et al., 1995), one possibility is that the effects of notochord on neural crest formation are mediated by these BMP-signaling inhibitors.

We have examined whether Noggin-secreting cells are able to mimic the notochord's ability to suppress neural crest formation and *Slug* expression. Our results show that Noggin-expressing cells are able to down-regulate *Slug* expression and inhibit neural crest cell emigration. However, the period of sensitivity to Noggin is different from that of sensitivity to Shh. If injected into the lumen of a closing neural tube (approximately mid-segmental plate level), Noggin-expressing cells are able to suppress both *Slug* expression and neural crest cell emigration. In contrast, cells injected at rostral segmental plate levels fail to suppress neural crest migration.

Injections of Noggin cells into open neural plate fall into two categories. When injected cells became displaced ventrally as a consequence of neural tube closure, no effects were observed on *Slug* expression or neural crest migration. However, when Noggin-expressing cells remained in close contact with the neural folds after tube closure, *Slug* expression and neural crest emigration were prevented. One possibility that can account for these differences is that Noggin-expressing cells require prolonged contact with neural fold cells to suppress neural crest formation. If the cells became displaced ventrally, the estimated 4-6 hours of contact would be insufficient to have an effect. Because *Slug* expression can be down-regulated by Noggin-expressing cells within a few hours (our unpublished observation), our favored explanation is that neural crest formation is sensitive to Noggin only at or shortly after neural tube closure. Thus, cells injected into the open neural plate can only affect neural crest formation if they come to lie dorsally at this later sensitive stage. Further evidence in support of the latter possibility comes from our in vitro studies, where we find that neural crest formation by cultured neural folds can be perturbed by Noggin only if administered between 5 and 10 hours of culture, corresponding precisely to the time at which neural tube closure would be occurring in vivo. Since BMP-4 expression at this time is limited to the dorsal neural tube, we propose that expression of BMPs by the prospective epidermis at open neural plate levels is not required for neural crest cell formation.

Noggin-expressing cells injected around the time of neural tube closure are able to down-regulate expression of BMP-4 itself, a finding consistent with the notion that levels of BMP-4 protein regulate BMP-4 gene expression. Notochord grafts adjacent to the open neural plate can also inhibit expression of BMP-4, but only when host embryos are incubated for 15-18 hours; in contrast, they fail to suppress BMP-4 expression if allowed to develop for shorter times. This finding suggests (i) that prolonged contact is required between notochord and neural folds to suppress BMP-4 expression, perhaps due to low levels of Noggin or Chordin protein within the notochord, and/or (ii) that the period of development during which BMP-4 expression is sensitive to Chordin or Noggin is after neural tube closure.

Injection of cells producing BMP-4 into the neural tube up-regulates expression of *Wnt*-1 and *Wnt-3* a (Marcelle et al., 1997). Conversely, the present study shows that injection of Noggin-producing cells into the neural tube down-regulates the expression of *Wnt-1*. Thus, BMPs may be important for induction or maintenance of *Wnt* expression. Because the *Wnt-1/3a* double mutant mouse has a reduction in the neural crest population (Ikeya et al., 1997), it is tempting to speculate that BMPs in the dorsal neural tube may be important for a *Wnt*-mediated expansion or maintenance of the neural crest population. In contrast, it is clear that *Wnt-1* and *Wnt-3a* are not required for *Slug* expression since expression of *Slug* can be induced in neural plate-epidermis recombinants at stages when identical recombinants fail to express either *Wnt* gene (Dickinson et al., 1995).

Our results raise new questions about the nature and timing of the neural crest-inducing signal that arises from the epidermis. We previously showed that interactions between naïve neural plate and non-neural ectoderm result in the formation of neural crest cells (Selleck et al., 1995; Dickinson et al., 1995). Open neural plate from stage 8-10 embryos (similar to the stages used in this study) as well as prospective neural plates from stage 4 embryos were able to form HNK-1 and *Slug*-positive cells when juxtaposed with non-neural ectoderm (Dickinson et al., 1995). Although BMP-4 has been shown to be able to substitute for epidermis in inducing neural crest cells from isolated neural plates (Liem et al., 1995), the current studies suggest that BMP-4 is unlikely to be the epidermal inducer. One possibility is that interactions between ectoderm and neural plate induce BMP, which in turn induces neural crest secondarily.

The role of *Slug* in neural crest formation

Little is known about the function of the transcription factor *Slug*. Recent studies of *Slug* in a rat bladder carcinoma cell line (Savagner et al., 1997) point to a role in regulating desmosome assembly in these epithelial cells. Notochord grafts and Shh- and Noggin-cell injections are able to down-regulate *Slug* expression at a variety of time points, even at relatively late developmental times, around the time of neural crest emigration from the neural tube. In contrast, an effect of these treatments on neural crest cell emigration is seen only at earlier stages of neurulation, prior to, and around the time of, neural tube closure. This raises the interesting question of the role of *Slug* in neural crest induction. One possibility is that *Slug* is required for neural crest formation, but only during a short time just after neural tube closure (when transcripts first appear in the dorsal neural tube). In support of this idea, antisense oligonucleotide knock-out of *Slug* transcripts in the early chick embryo blocks neural crest emigration (Nieto et al., 1994). Although after this time 'window' *Slug* is down-regulated by implantation of notochords, Noggin or Shh cells, our results suggest that this inhibition of *Slug* expression has no observable effect on neural crest formation. A second possibility is that *Slug* is required for the formation of only a subset of neural crest cells, the absence of which we could not detect in our DiI migration assay. Finally, we cannot rule out the possibility that, though a useful marker, *Slug* is not required for neural crest formation and that down-regulation has no effect on neural crest production.

Conclusion

The present experiments have clarified the temporal sequence of neural crest formation by grafting the notochord or some of its molecular constituents into the open neural plate. The goal was to define the timing and some of the molecular events that are important for establishing dorsal fates in the neural tube. The finding that ventralizing signals can override dorsalizing signals if presented at the beginning of neurulation suggest that specification of the neural crest is not complete at the open neural plate stage. The observed dominance of ventralizing signals over dorsalizing signals may reflect the time at which notochordal and epidermal signals are 'read' by the neuroepithelium. We have used the potent ventralizing agent Sonic hedgehog and the BMP-inhibitor Noggin to challenge the ability of the neural folds to form neural crest cells. The results suggest that neural crest specification is not a single-step process, but rather is a continuous, multiphasic process. We propose that neural crest formation consists of: (1) an initial phase at open neural plate levels (towards the caudal end of the segmental plate), when neural crest formation is sensitive to Shh, but cannot be inhibited by the BMP-signaling antagonist, Noggin; (2) a subsequent BMP-mediated phase around the time of neural tube closure (a position adjacent to the mid-segmental plate), corresponding to the time when BMP-4 is expressed in the dorsal neural tube but not the epidermis; and (3) a later pre-migratory phase (corresponding to a level adjacent to the rostral segmental plate) which is refractory to both Shh and Noggin. Thus, generation of the neural crest may involve a variety of processes and molecular signals. Clearly, induction of the neural crest is tightly linked to, and may involve some of the same signals as, dorsoventral patterning of the neural tube. In fact, a competition between dorsal and ventral signals may be involved in the initial step in neural crest induction. The present study raises new questions as to the relationship between neural plate-epidermis interactions and BMP expression in the dorsal neural tube, and the identity of the ectodermal signals involved in neural crest induction.

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